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Type 2 immunity is controlled by IL-4/IL-13 expression in hematopoietic non-eosinophil cells of the innate immune system

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Nippostrongylus brasiliensis infection and ovalbumin-induced allergic lung pathology are highly interleukin (IL)-4/IL-13 dependent, but the contributions of IL-4/IL-13 from adaptive (T helper [Th]2 cells) and innate (eosinophil, basophils, and mast cells) immune cells remain unknown. Although required for immunoglobulin (Ig)E induction, IL-4/IL-13 from Th2 cells was not required for worm expulsion, tissue inflammation, or airway hyperactivity. In contrast, innate hematopoietic cell-derived IL-4/IL-13 was dispensable for Th2 cell differentiation in lymph nodes but required for effector cell recruitment and tissue responses. Eosinophils were not required for primary immune responses. Thus, components of type 2 immunity mediated by IL-4/IL-13 are partitioned between T cell-dependent IgE and an innate non-eosinophil tissue component, suggesting new strategies for interventions in allergic immunity.

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Abbreviation used: tg, transgenic.

Type 2 immune responses are induced by parasitic helminthes and are associated with atopic diseases, such as allergy and asthma. Hallmarks of type 2 immune responses include elevated serum IgE, infiltration of affected tissues by Th2 cells, eosinophils and basophils, and epithelial cell differentiation to mucus-secreting, hyperproliferative states (1–3). IL-4 and IL-13 are critically involved in the onset and effector phase of type 2 immune responses in vivo. IL-4/IL-13-deficient mice show drastically delayed expulsion of helminth parasites (4), whereas IL-4 or IL-13 administration to CD4-depleted or genetically T cell-deficient mice is sufficient to expel worms (5, 6). In addition, IL-13 and IL-4, when overexpressed in the lungs of mice or administered exogenously, induce inflammatory features of asthma, including eosinophil infiltration, mucus cell hyperplasia, and increases in baseline airway reactivity (7, 8). Th2 cells are thought to be the main source for IL-4/IL-13 during the effector phase of type 2 immune responses and have been identified in proximity to parasitic worms in affected tissues (9). Innate cell sources of IL-4/IL-13, such as eosinophils, basophils, or mast cells, have been proposed to help initiate the early phase of Th2

differentiation, based in part on the ease with which naive T cells are differentiated to Th2 cells in the presence of IL-4 in vitro. However, IL-4/IL-13 is clearly not essential for Th2 cell differentiation in vivo because both IL-4R α - and STAT6-deficient mice have been shown to generate substantial numbers of Th2 cells in the draining lymph nodes during infection with helminth or protozoan parasites (6, 10–13). Importantly, the stabilization of Th2 differentiation and the formation of Th2 memory cells may be dependent on IL-4 because secondary immune responses are greatly impaired in STAT6-deficient mice (11).

An understanding of the relative roles of IL-4/IL-13 expressed from Th2 cells as compared from cells of the innate immune system will be important in devising strategies to augment or impede allergic immune responses. Here, we used sensitive IL-4 reporter mice (4get mice) in combination with adoptive cell transfers, bone marrow chimeras, and hypereosinophilic and eosinophil-deficient mice to analyze critically the contributions made by various IL-4/IL-13-producing cells in the immune response against *Nippostrongylus brasiliensis* and in the generation of OVA-induced allergic lung pathology. The findings reveal an unappreciated partitioning of IL-4/IL-13-mediated

The online version of this article contains supplemental material.

effector function between adaptive Th2 cells and innate immune cells.

RESULTS

Innate IL-4/IL-13 is required for worm expulsion

Infection of mice with *N. brasiliensis* induces a strong type 2 immune response in the lung and small intestine. Expulsion of intestinal adult worms is critically dependent on T cells and IL-4/IL-13, although the cellular source of these cytokines has not been determined (5). To analyze whether expression of IL-4/IL-13 was required from T cells or non-T cells, RAG-deficient mice were reconstituted with 10^7 CD4 T cells purified from STAT6- or IL-4/IL-13-deficient mice. 4 d after T cell reconstitution, mice were infected with *N. brasiliensis* and the burden of adult worms in the intestines was analyzed 9 d later. In contrast to nonreconstituted RAG-deficient mice, which had abundant intestinal worms, mice reconstituted with either STAT6- or IL-4/IL-13-deficient CD4 T cells expelled the worms, indicating that IL-4/IL-13 from CD4 T cells was not required for worm expulsion (Fig. 1 A). However, expression of these cytokines from another cell type was essential because infected mice could not expel the worms in the complete absence of IL-4/IL-13, as shown previously (14). To assess whether innate cell-derived IL-4/IL-13 was required or simply sufficient for worm expulsion, similar experiments were performed by reconstituting TCR- α -deficient (which lack T cells but not B cells) or IL-4/IL-13/TCR- α -deficient mice with 10^7 purified CD4 T cells from WT or IL-4/IL-13-deficient mice. After 4 d, reconsti-

tuted mice were infected with *N. brasiliensis* and analyzed for class switching to IgE and worm expulsion on day 10 after infection. IgE production was highly dependent on IL-4/IL-13 produced by CD4 T cells and could not be made in its absence. In contrast, worm expulsion was highly dependent on IL-4/IL-13 from non-T cells, and worms could not be expelled in their absence, even when IL-4/IL-13 was present in WT CD4 T cells (Fig. 1 B). Therefore, the cytokines IL-4/IL-13 required for intestinal worm expulsion have to be expressed in non-T cells.

Innate IL-4/IL-13 is required for Th2 cell accumulation in the lung

To analyze whether innate IL-4/IL-13 was also required for T cell differentiation to Th2 cells in draining lymph nodes or for their accumulation at affected tissue sites, 10^7 purified CD4 T cells from IL-4 reporter mice (4get mice) were transferred into either TCR- α -deficient or IL-4/IL-13/TCR- α -deficient mice, which were then infected and analyzed 9 d later at the peak of the immune response for the presence of Th2 cells in the lung or draining paratracheal lymph nodes. Th2 cells could be generated efficiently in draining lymph nodes in the absence of IL-4/IL-13 from non-T cells because both groups of reconstituted mice generated $\sim 20\%$ Th2 cells (GFP⁺) among total CD4 T cells (Fig. 2 A). However, accumulation of Th2 cells in the infected lung tissues was 10-fold reduced in the IL-4/IL-13/TCR- α -deficient recipient mice as compared with the TCR- α -deficient recipient mice. Because adoptive transfer of T cells into T cell-deficient

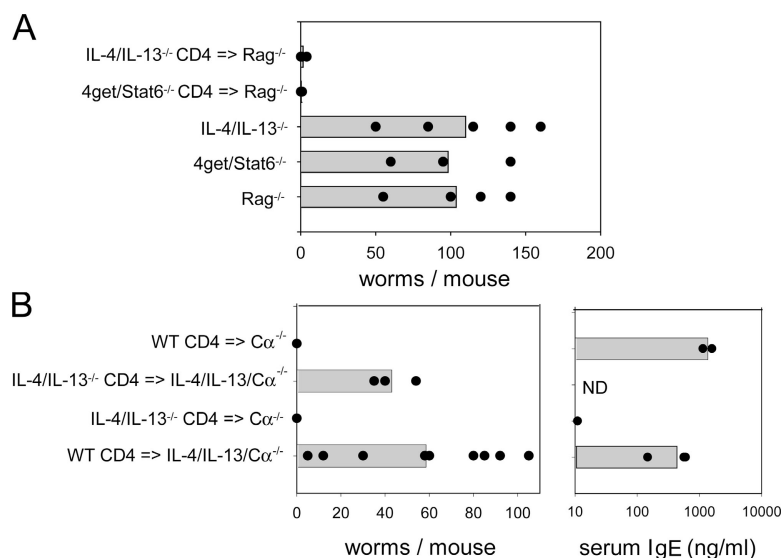


Figure 1. Worm expulsion requires expression of IL-4/IL-13 from cells of the innate immune system. (A) 10^7 MACS-purified naive CD4 T cells from IL-4/IL-13- or 4get/STAT6-deficient mice were adoptively transferred into RAG-deficient mice. Transferred mice and indicated control mice were infected with *N. brasiliensis* 4 d after T cell transfer as described in Materials and methods. Adult worms were counted 9 d after infection in the small intestine. Three to five mice per group were

analyzed. (B) 10^7 MACS-purified naive CD4 T cells from BALB/c or IL-4/IL-13-deficient mice were adoptively transferred into TCR- α - or IL-4/IL-13/TCR- α -deficient mice. Reconstituted mice were infected with *N. brasiliensis* 4 d after transfer and analyzed for worm expulsion and serum IgE levels on day 9 after infection. 3–9 mice per group were analyzed. ND, not detectable. Both experiments were repeated once with similar results.

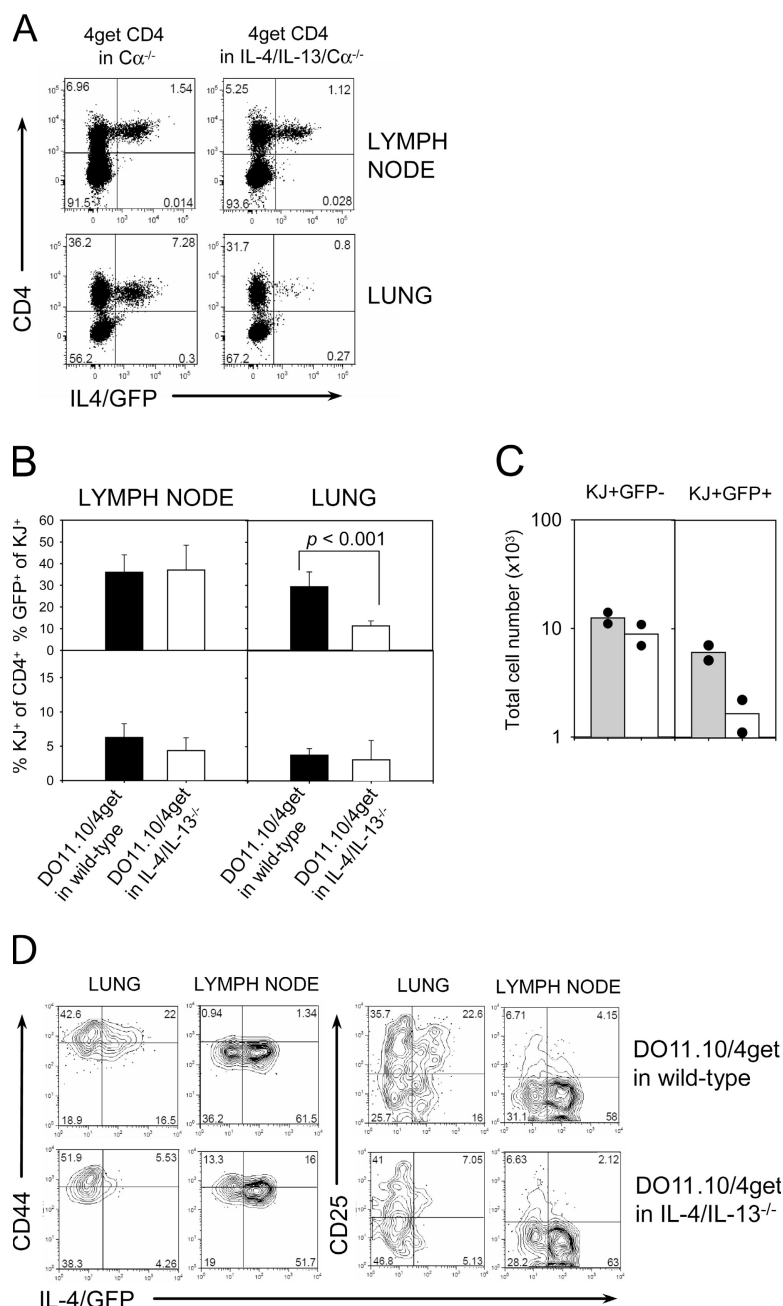


Figure 2. Accumulation of Th2 cells in the lung requires IL-4/IL-13 production from cells of the innate immune system. (A) 10^7 MACS-purified naive CD4 T cells from 4get mice were transferred into TCR- $C\alpha^{-/-}$ ($C\alpha^{-/-}$) or IL-4/IL-13/TCR- $C\alpha^{-/-}$ (IL-4/IL-13/ $C\alpha^{-/-}$) mice, and the groups were infected with *N. brasiliensis* 4 d later. The frequency of Th2 cells in the lung or paratracheal lymph nodes was analyzed by flow cytometry on day 9 after infection. Expression of GFP indicates the expression of IL-4 in these reporter mice. One representative experiment of four is shown. (B) 10^6 naive KJ1-26⁺ CD4 T cells from DO11.10/4get/TCR- $C\alpha^{-/-}$ mice were transferred into BALB/c or IL-4/IL-13-deficient mice, which were inoculated with *N. brasiliensis* and OVA 1 d after transfer, challenged intranasally with OVA on day 6, and analyzed by flow

cytometry on day 9. Bars indicate the frequency of IL-4/GFP-expressing cells among antigen-specific KJ1-26⁺ cells and the frequency of KJ1-26⁺ cells among total CD4 T cells in the lung or lymph nodes of WT (WT) recipients (closed bars) or IL-4/IL-13-deficient recipients (open bars). The experiment shows the combined results from two independent experiments with a total of five mice per group. Error bars indicate the standard deviation. The p-value was determined by Student's *t* test. (C) Total numbers of clonotypic KJ1-26⁺ cells in the lungs of reconstituted BALB/c mice (closed bars) or IL-4/IL-13-deficient mice (open bars). (D) Activation of antigen-specific T cells as assessed by expression of CD44 and CD25 in the lung and lymph node.

mice can lead to homeostatic proliferation and differentiation of donor T cells, which might influence the outcome of the experiment, similar experiments were performed in non-lymphopenic mice after reconstitution with antigen-specific CD4 T cells. WT or IL-4/IL-13-deficient BALB/c mice were given 10^6 OVA-specific CD4 T cells from 4get/DO11.10 TCR transgenic (tg) mice. Recipient mice were infected with 500 *N. brasiliensis* L3 larvae in 200 μ l of a 500 μ g/ml OVA protein in saline solution, challenged on day 6 after infection by intranasal administration of 1 mg OVA protein, and analyzed 3 d later. OVA-specific CD4 T cells differentiated efficiently into Th2 cells in the draining lymph nodes of the lung independently of the presence or absence of IL-4/IL-13 in the host (Fig. 2 B). However, infiltration of transferred OVA-specific Th2 cells into the lungs of IL-4/IL-13-deficient recipient mice was reduced by 70% as compared with WT recipients. Total numbers of antigen-specific, GFP⁺ CD4 T cells were not significantly different in the lungs of the two groups of reconstituted mice, but the numbers of GFP⁺ CD4 T cells were substantially reduced in the tissue, but not the draining lymph nodes (Fig. 2 C). Reduced numbers of GFP⁺ cells were not due to a general defect of T cell activation in the lung of IL-4/IL-13-deficient recipients because up-regulation of CD44 and CD25 was comparable in WT and IL-4/IL-13-deficient mice (Fig. 2 D). Collec-

tively, these experiments indicate that expression of IL-4/IL-13 from innate cells is not required for Th2 cell differentiation in lymph nodes but is critically required for optimal accumulation of Th2 cells at sites of infection.

Eosinophils and basophils constitute the main IL-4/IL-13-producing cells of the innate immune system after *N. brasiliensis* infection or OVA-induced lung sensitization

A recent report indicated that muscle cells can express IL-4 during myotube regeneration (15). Thus, nonhematopoietic cells might contribute to the non-T cell-mediated IL-4/IL-13 required for efficient type 2 immunity. To assess this possibility, bone marrow chimeras were generated to analyze whether expression of IL-4/IL-13 from non-bone marrow-derived cells could contribute to effector cell recruitment or worm expulsion during *N. brasiliensis* infection. WT bone marrow from 4get mice was transferred into lethally irradiated WT, IL-4-deficient, or IL-4/IL-13-deficient mice. 8 wk after reconstitution, mice were infected with *N. brasiliensis* and analyzed 9 d later for effector cell recruitment and worm expulsion. All of the mice in each group completely eliminated the parasites (not depicted) and showed comparable numbers of infiltrating effector cells in the lung, indicating that non-bone marrow-derived IL-4/IL-13 plays no significant role in the immune response against *N. brasiliensis*.

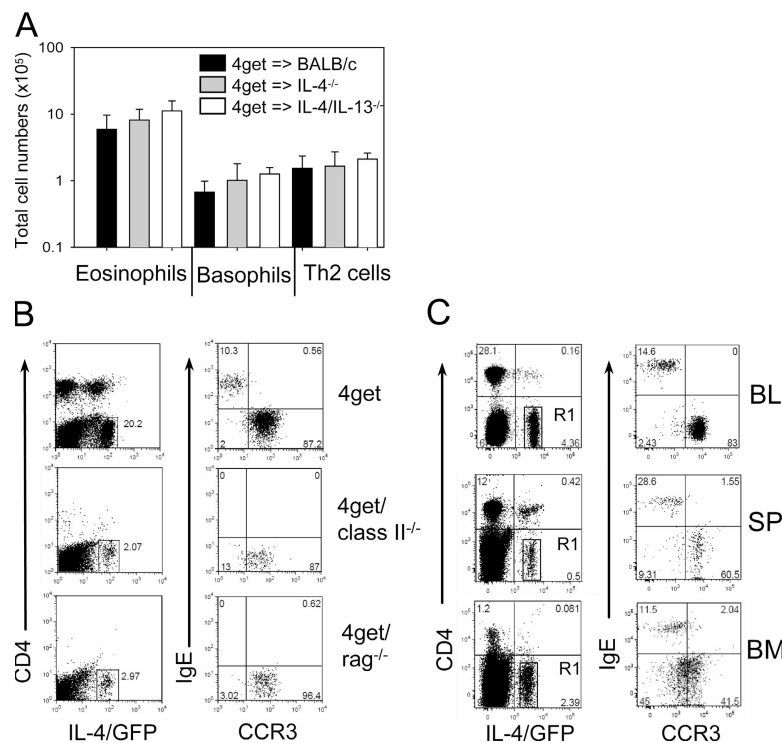


Figure 3. Bone marrow-derived cells and CD4 T cells regulate type 2 immunity in vivo. (A) Bone marrow cells from 4get mice were transferred into lethally irradiated WT, IL-4-deficient, or IL-4/IL-13-deficient recipient mice. 8 wk after reconstitution, three mice per group were infected with *N. brasiliensis* and analyzed on day 9 after infection by four-color flow cytometry for the presence of Th2 cells, eosinophils, and

basophils in the lung after staining for CD4, IgE, and CCR3. Error bars indicate the standard deviation. (B) Single cell suspensions of the lung of 4get, 4get/MHC class II^{-/-}, or 4get/RAG^{-/-} mice were stained for CD4, IgE, and CCR3 and analyzed 9 d after *N. brasiliensis* infection by flow cytometry. (C) The blood, spleen, and bone marrow of naive 4get mice were analyzed by flow cytometry using the markers indicated above.

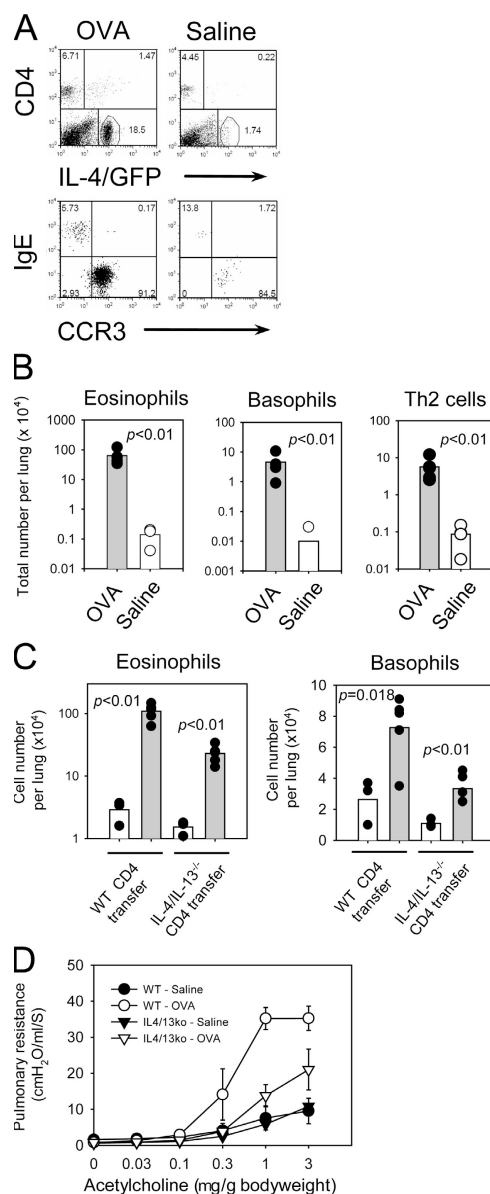


Figure 4. Eosinophils and basophils are the major innate IL-4-expressing cells recruited to the lung during OVA-induced lung inflammation. (A) 4get mice were primed with OVA or saline and challenged for three consecutive days with OVA as described in Materials and methods. Single cell suspensions of the lung were stained and analyzed as described in Fig. 3. (B) Total numbers of eosinophils, basophils, and Th2 cells in the lung of five OVA-challenged mice or three saline-treated control mice were calculated based on the frequency of each population among total cells in the lung. (C) 4get/RAG^{-/-} mice were reconstituted with 10^7 CD4 T cells from BALB/c or IL-4/IL-13-deficient mice. A group of five mice was sensitized and challenged with OVA (closed bars), whereas another group of three mice was treated with saline (open bars). The total number of eosinophils and basophils in the lung was determined 1 d after the last challenge. p-values were determined by Student's *t* test and are indicated in the figure. (D) Pulmonary resistance was determined in both groups of mice in anesthetized mice by injecting increasing doses of acetylcholine 1 d after the last challenge of 4get/RAG^{-/-} mice reconstituted with BALB/c (circles) or IL-4/IL-13-deficient (triangles) T cells. Error bars indicate the standard deviation. $P < 0.001$ for WT saline versus OVA, and

(Fig. 3 A). Thus, elimination of parasitic worms and accumulation of Th2 cells in the lung are dependent on IL-4/IL-13 expressed from a hematopoietic cell of the innate immune system.

RAG-deficient mice show drastically impaired recruitment of eosinophils and basophils to tissues during *N. brasiliensis* infection (3). However, transfer of IL-4/IL-13-deficient CD4 T cells restored effector cell recruitment, indicating that IL-4/IL-13 production from Th2 cells was not required. To assess whether this function was specific to CD4 T cells, or whether CD8 T cells could subserve the same effector function, MHC class II-deficient 4get mice were infected with *N. brasiliensis*. Analysis of the lungs after 9 d demonstrated that CD4 T cells were absolutely required for effector cell recruitment because MHC class II-deficient mice had defects in eosinophil and basophil recruitment that were comparable to RAG-deficient mice (Fig. 3 B). Thus, CD8 T cells and NKT cells, which are intact in MHC class II-deficient mice, cannot subserve the effector cell tissue-recruiting function of CD4 T cells in type 2 immunity.

Mast cells, eosinophils, and basophils can produce IL-4/IL-13 and might be considered candidates for innate cells required to mediate IL-4/IL-13-dependent type 2 immune responses in vivo. These three cell types are constitutively fluorescent in 4get mice, likely reflecting the positioning of the GFP reporter downstream of an IRES element mutated to facilitate translation and consistent with an accessible chromatin configuration at the IL-4 locus, which facilitates the rapid production of IL-4 during the early phase of infection (3, 16–18). Basophils and eosinophils are readily distinguished in the blood, spleen, and bone marrow of uninfected 4get mice by their GFP expression together with staining for CCR3 on eosinophils and IgE bound to the high-affinity FcεRI receptor on basophils (Fig. 3 C). In bone marrow, eosinophils express a range of CCR3 consistent with their immature phenotype.

N. brasiliensis infection leads to a marked increase of eosinophils and basophils in tissues, which, with Th2 cells, comprised all of the IL-4-producing cells (3). To assess whether this reflected the stereotyped nature of type 2 immune responses, 4get mice were sensitized with OVA and the lungs were examined after repeated airway administration of OVA to induce allergic inflammation. Indeed, as in the *Nippostrongylus* model, basophils and eosinophils were the predominant innate IL-4-expressing cells that were recruited to the lung after antigen challenge of sensitized mice (Fig. 4 A). Eosinophils were present in 10-fold larger numbers than basophils or Th2 cells (Fig. 4 B). The IgE-binding cells were c-kit⁻, FSC/SSC^{low}, consistent with their identification as basophils rather than mast cells, which are induced poorly in the lung during *Nippostrongylus* infection (3) or after acute OVA sensitization and challenge (19).

$P = 0.027$ for IL-4/IL-13-deficient saline versus OVA. The experiment was performed twice with similar results.

T cell-derived IL-4/IL-13 amplifies but is not required for OVA-induced allergic airways disease

As in *Nippostrongylus* infection, IL-4/IL-13 plays an essential role in the development of the inflammatory immune response, mucus secretion, and airway hyperreactivity. To assess whether IL-4/IL-13 from CD4 T cells is required for effector cell recruitment and airway hyperreactivity, 4get/RAG-deficient mice were reconstituted with 10^7 CD4 T cells purified from IL-4/IL-13-deficient or WT mice. After i.p. administration of saline or OVA, animals were subjected to repeated intranasal OVA challenge, and then analyzed for effector cell recruitment to the lung and for airway hyperreactivity to increasing doses of acetylcholine (Fig. 4, C and D). Although WT CD4 T cells reconstituted eosinophil and basophil recruitment together with airway hyperreactivity to allergen challenge, IL-4/IL-13-deficient CD4 T cells also mediated significant eosinophil and basophil recruitment as well as airway hyperreactivity (Fig. 4, C and D). As the OVA model relies on repetitive administration of the allergen, this understandably favors the amplification of pathology by the expanded T cell population. Importantly, however, innate, non-T cell-derived IL-4/IL-13 is sufficient to mediate the effector phase of allergic lung disease as long as CD4 T cells are present to facilitate their recruitment and/or organization in tissues.

High numbers of eosinophils attenuate a type 2 immune response

The relative contribution of basophils for Th2 cell or eosinophil accumulation in the lung and worm expulsion from the intestine could not be analyzed directly because basophils could not be isolated in sufficient numbers for adoptive transfer experiments and basophil-deficient mice are not currently available. However, a potential role of eosinophils could be analyzed using IL-5tg mice (20), which produce massive numbers of eosinophils, and Δ dblGATA mice (21), which lack eosinophils due to a mutation of a GATA-1 binding site in the *gata-1* promoter. Eosinophils were isolated from the peritoneum of IL-5tg mice, labeled with CFSE, and transferred i.v. to recipient mice, which had been infected with *N. brasiliensis* 1 d previously. 16 h later, the transferred eosinophils had migrated to the lung, where they constituted 2.6% of total cells (Fig. S1 A, available at <http://www.jem.org/cgi/content/full/jem.20052448/DC1>). The eosinophil numbers declined to undetectable levels in the blood and lung by day 3 after transfer (not depicted). This adoptive transfer system was then used repeatedly to transfer eosinophils from IL-5tg mice into IL-4/IL-13/TCR-C α -deficient mice, which had been reconstituted with 10^7 purified CD4 T cells from 4get mice. In this way, only eosinophils could provide the innate source of IL-4/IL-13 required for the

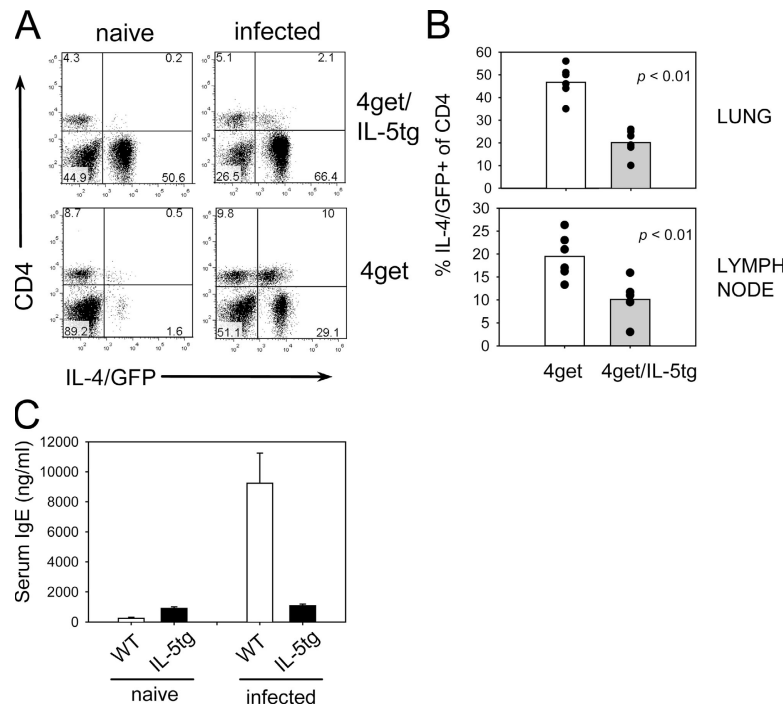


Figure 5. Attenuated induction of type 2 immunity in IL-5tg mice. (A) The lungs of normal (4get) or IL-5tg 4get (4get/IL-5) mice were analyzed by flow cytometry before (naive) or 9 d after (infected) *N. brasiliensis* infection by staining with anti-CD4 to identify Th2 cells (CD4⁺IL-4/GFP⁺). (B) Frequency of Th2 cells in paratracheal lymph nodes and lungs of 4get (open bar) and 4get/IL-5tg (closed bar) mice on day 9

after infection. (C) Analysis of serum IgE levels before (naive) or 9 d after (infected) infection of 4get or 4get/IL-5tg mice. Error bars indicate the standard deviation. (B and C) Combined results from three independent experiments with a total of six mice per group. p-values were determined with Student's *t* test.

effector phase of type 2 immunity. Eosinophils were transferred on days 1, 4, and 6 after infection to account for the relatively short half-life of these cells, and infected mice were analyzed on day 9. Despite the capacity to restore the lung with IL-4/IL-13-competent eosinophils, however, the adoptive transfer system used here was not sufficient to increase the number of Th2 cells in the lung or to expel the intestinal parasites (Fig. S1B).

Next, 4get/IL-5tg mice were infected with *N. brasiliensis* to determine whether constitutively high levels of eosinophils would increase the number of Th2 cells in the lung. As compared with WT mice, 4get/IL-5tg mice showed reduced Th2 cell differentiation in the draining lymph nodes and lung (Fig. 5, A and B), and no increase in serum IgE (Fig. 5 C). Presumably, these attenuated responses reflect the finding that IL-5tg mice are highly resistant to *N. brasiliensis* due to

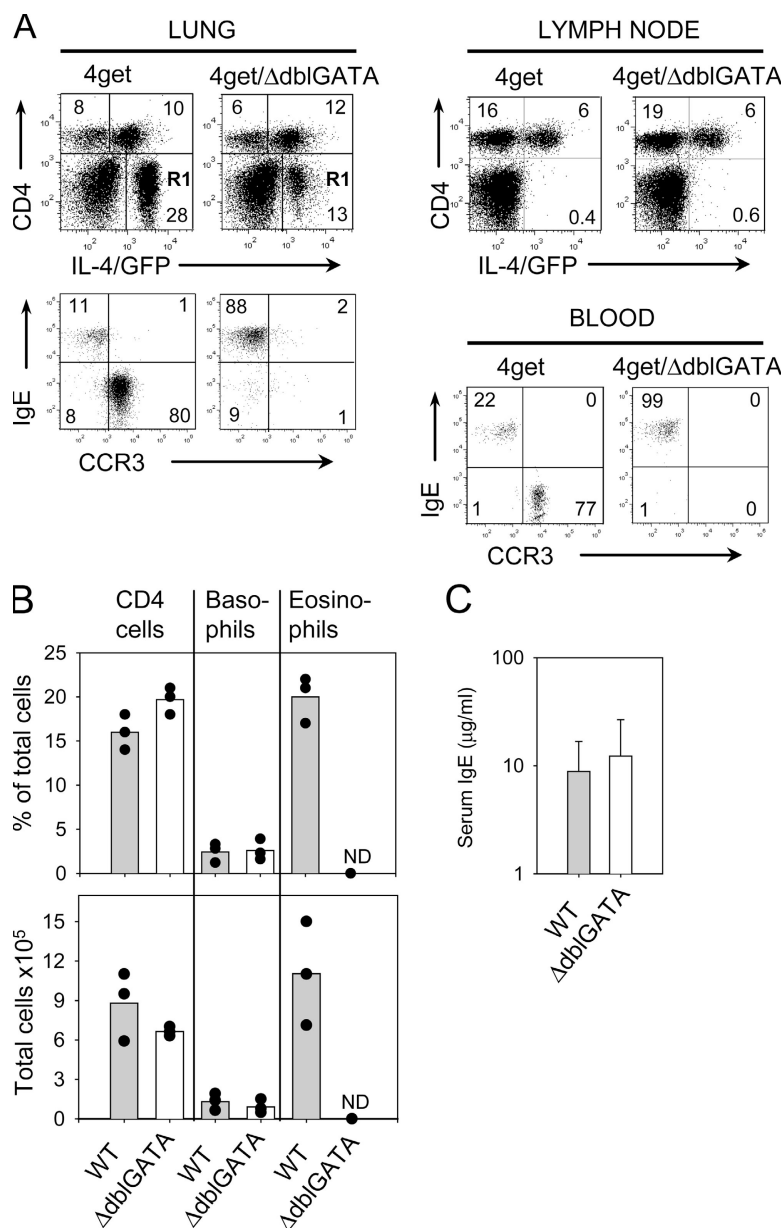


Figure 6. Eosinophils are not required for Th2 or basophil recruitment to the lung, increased serum IgE levels, or worm expulsion after *N. brasiliensis* infection. (A) WT 4get or eosinophil-deficient 4get/ Δ dblGATA mice were infected with *N. brasiliensis* and analyzed 9 d later for Th2 cell polarization in paratracheal lymph nodes, the frequency of eosinophils and basophils in the blood, and infiltration of CD4 T cells, basophils, and eosinophils into the lung parenchyma by flow cytometry.

The dot plots from the lung and lymph node are gated on total live cells, and the lung and blood samples are gated on CD4-GFP⁺ cells. (B) Frequency and total cell number of eosinophils, basophils, and Th2 cells in the lungs of BALB/c (WT) and Δ dblGATA mice on day 9 after infection. (C) Total serum IgE from BALB/c (WT) and Δ dblGATA mice (three mice per group) determined by ELISA on day 9 after infection. Error bars indicate the standard deviation.

immediate killing of migrating larvae by eosinophils at the site of infection (22, 23). Intriguingly, despite injection of the same amounts of helminth antigen in IL-5tg mice as in the WT mice, the type 2 immune response was highly abrogated, indicating that parasite migration and shedding of larval antigens might be required to elicit a strong type 2 immune response in vivo. Alternatively, eosinophils may degrade antigen such that antigen presentation by dendritic cells in the draining lymph nodes is compromised.

Eosinophils are not required for accumulation of Th2 cells in the lung, but contribute to protection against secondary helminth infection

To assess whether IL-4/IL-13 produced by eosinophils was required for worm expulsion or to mediate the accumulation of Th2 cells and basophils in the lung, Δ dblGATA mice were crossed with 4get mice (21). The increased sensitivity afforded by the GFP marker in eosinophils confirmed the complete absence of eosinophils in these mice, with no compromise of the basophil lineage (not depicted). The mice were infected with *N. brasiliensis* and analyzed at the peak of the response on day 9, which demonstrated no deficiency in Th2 generation in lymph nodes, in the percentages or numbers of Th2 effector cells and basophils recruited to the lung, or in the capacity to expel worms or generate IgE (Fig. 6, A–C, and not depicted). No residual eosinophils were detectable in the blood or lung of the mutant mice. Thus, eosinophils are not required as a source of IL-4/IL-13 to mediate Th2 cell accumulation or worm expulsion during a primary type 2 immune response against *N. brasiliensis*.

In contrast to a primary infection, where worm expulsion is clearly CD4 T cell dependent, secondary responses against worms, including efficient elimination, can occur in the absence of CD4 T cells (24). To address the possibility that eosinophils, which are highly abundant in chronic migratory parasitic infections, might play a crucial role during secondary memory responses against helminth parasites, we infected WT and Δ dblGATA mice with *N. brasiliensis*, allowed the infection to resolve, and rechallenged the mice 4 wk later. A designated cohort of the rechallenged mice was treated with anti-CD4 GK1.5 monoclonal antibody to deplete CD4 T cells 3 d before the secondary infection. Mice were analyzed 7 d after the secondary infection for intestinal worm expulsion (Fig. 7). As expected, mice that were not depleted of CD4 cells cleared the infection, and this was largely comparable in the absence of eosinophils (a single eosinophil-deficient animal had identifiable worms). Depletion of CD4 T cells during the secondary challenge, however, resulted in a statistically significant three- to fourfold larger number of worms in the absence, as compared with the presence, of eosinophils, consistent with an important role for these cells during secondary responses to migratory helminthes.

DISCUSSION

The murine models of *N. brasiliensis* infection and OVA-induced lung disease represent highly characterized models of

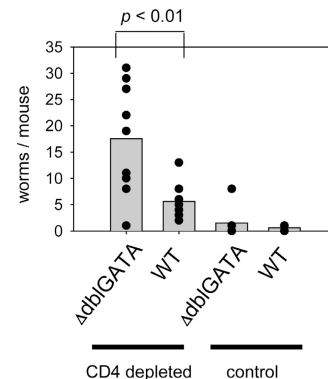


Figure 7. Eosinophils contribute to a secondary immune response against *N. brasiliensis* larvae. 4get or 4get/ Δ dblGATA mice were infected with *N. brasiliensis*, rechallenged 5 wk later, and analyzed for worm expulsion on day 7 after secondary infection. One set of mice from each group was depleted of CD4 cells 3 d before the second infection. The graph shows the combined results from two independent experiments. The p-value was determined by Student's *t* test.

type 2 immunity; both are critically dependent on CD4 T cells and IL-4/IL-13 as well as the shared components of their signaling, IL-4R α and STAT6 (2, 25). One reflects the response to a complex array of diverse parasite antigens typically assessed at days 9 or 10, whereas the other reflects the focused response to a singular protein antigen challenge in the lung mucosa, typically generated over a period of several weeks. Despite these differences, the studies here reveal a highly stereotyped tissue response characterized by infiltrations of Th2 cells, eosinophils, and basophils. Th2 cell differentiation, as assessed by activation of IL-4 expression in lymph nodes, was independent of IL-4/IL-13 from other cell types, in support of prior studies (6, 10, 11). Although CD4 T cells were necessary and sufficient to mediate the infiltration of effector cells into target tissues, IL-4/IL-13 produced by Th2 cells was neither required nor sufficient for this process. Instead, tissue infiltration by effector cells was dependent on IL-4/IL-13 produced by innate immune cells, whereas IL-4/IL-13 produced by Th2 cells was required for IgE production. Tissue infiltration by Th2 cells, eosinophils, and basophils was tightly coordinated with effective physiologic responses, including intestinal worm expulsion and induction of airway hyperreactivity, which could be mediated by IL-4/IL-13 derived solely from innate cells. These findings force a reevaluation of the mechanisms by which type 2 immunity is established and reveal previously unrecognized cellular interactions between innate and adaptive cells.

The source of IL-4, which effectively mediates Th2 differentiation in vitro and supports Th2 differentiation in vivo, has been long debated, with various studies supporting roles for autocrine IL-4 production by CD4 T cells themselves (26, 27) or IL-4 production by a variety of other cell types (28, 29). As assessed by activation of the GFP marker in the IL-4 locus, neither IL-4 nor IL-13 from other cell types is required for differentiation of Th2 cells in vivo (Fig. 2 A).

The dense deficit in mediating type 2 immunity in IL-4/IL-13-, IL-4R α -, and STAT6-deficient mice is thus explained not by an inability to produce Th2 cells, but by the inability of IL-4/IL-13-producing effector cells to enter target tissues where they mediate the diverse biologic responses required to expel worms or generate airway hyperreactivity. The accumulation of the stereotyped tissue cellular response of type 2 immunity—Th2 cells, eosinophils, and basophils—was dependent on CD4 T cells but also on IL-4/IL-13 produced by bone marrow-derived innate immune cells. In the absence of either CD4 T cells or innate IL-4/IL-13, effector biology was lost. Although prior studies using *N. brasiliensis* have called attention to the requirement for IL-4R α expression on non-bone marrow-derived cells to effect tissue responses, including worm expulsion (30), our studies demonstrate that the source of IL-4/IL-13 required to drive these complex responses actually derives from innate immune cells rather than Th2 cells. CD4 T cell-derived IL-4/IL-13 amplified the lung inflammation that occurred after repetitive OVA stimulation, suggesting that T cells can amplify these innate responses with continued challenge and expansion, but innate cell IL-4/IL-13 was clearly capable of inducing allergic lung disease mediated by IL-4/IL-13-deficient T cells. Prior studies using *Brugia malayi* demonstrated that immunity could be mediated by adoptively transferred IL-4- or IL-4R α -deficient CD4 T cells into T cell-deficient recipients (31), but we extend these observations by the use of doubly IL-4/IL-13-deficient T cells. Additionally, basophil accumulation in the liver after *N. brasiliensis* infection was unaffected by the absence of IL-4 expression in T cells (32).

These findings extend our prior report (3) and find eosinophils and basophils to be the major infiltrating IL-4-expressing non-T cells in both allergic lung disease and in defense against migratory worms. Although mast cells accumulate under conditions of chronic mucosal stimulation (19), such as occurs in human asthma, neither mast cells nor IgE production are required for acute immunity against *N. brasiliensis* or OVA sensitization models using alum or repetitive antigen exposures (33–35). Extensive mast cell infiltration of airway smooth muscles, which has been documented in human asthma (36), does not occur in the mouse in these acute models.

The finding that eosinophils and basophils constitute the IL-4-expressing cells that accumulate in affected tissues forced us to consider the roles of these cells as a source of the innate IL-4/IL-13, which ultimately mediates type 2 immune effector function. Eosinophil function in parasitic disease has been much debated, despite the clear evidence that eosinophils are capable of direct toxicity to parasitic worms (37, 38). We document that eosinophils are completely and specifically deleted in Δ dblGATA mice, which contain a GATA-1 promoter mutation, corroborating prior studies (21), whereas all other IL-4-producing populations were unaffected (not depicted). *N. brasiliensis* infection of these eosinophil-deficient mice revealed no apparent role for eosinophils in either Th2 or basophil accumulation, IgE production, or

worm expulsion during primary infection, extending prior studies using IL-5-deficient animals, although residual eosinophils are present in these mice (39). Further, transfer of IL-4/IL-13-competent eosinophils into an IL-4/IL-13-deficient innate cell compartment, even in the presence of IL-4/IL-13-competent CD4 T cells, was not able to restore type 2 immunity (Fig. S1). Although negative experiments are difficult to interpret, we document reconstitution of transferred cells into relevant tissues. Further, if eosinophils were required to nucleate type 2 immunity in tissues, we would have anticipated greater responses in IL-5tg mice, which have numerous eosinophils in tissue. These mice, however, demonstrated attenuated type 2 immune responses after infection, as reflected by low serum IgE and a decrease in tissue-infiltrating cells, in agreement with prior findings (23). Indeed, we were able to document a significant role for eosinophils in secondary challenges with *N. brasiliensis* (Fig. 7), consistent with a role in attacking migratory larvae in tissues, and similar to findings reporting increased worm burdens of *Trichinella spiralis* in IL-5-deficient mice (40). Although the three- to fourfold reductions we document during secondary challenge seem modest, the contributions by eosinophils to control worm burden in endemic countries where reinfection takes place repeatedly over the human lifespan are likely considerable, and may significantly contribute to protection from helminth infections among patients with AIDS living in such endemic areas of the world.

Intriguingly, in both *N. brasiliensis* and OVA-induced airway disease, basophils constitute a significant population of IL-4-producing cells that are, as we demonstrate elsewhere, capable of entering tissues in the absence of STAT6-mediated signals (3). Thus, basophils can accumulate without IL-4/IL-13-mediated tissue-derived signals, although their recruitment is highly dependent on other signals provided by CD4 T cells (3, 32). Basophils have been reported to generate greater amounts of IL-4 than Th2 cells on a per-cell basis (41–43), and infiltration of lungs by basophils has been linked with severe and fatal human asthma (44, 45). Production of IL-4 by basophils has been documented in anti-IgD-primed mice, a potent type 2 immune stimulus (46), during secondary challenge with goat serum, although a critical role for these cells in primary type 2 immunity remains unproved. Indeed, even the precise lineage of basophils in hematopoiesis is unclear, and further work is justified in assessing the role of these cells in type 2 immune responses.

The major functional activity of Th2 cells in affected tissues was linked with the capacity to orchestrate the accumulation of eosinophils and basophils. Indeed, the accumulation of Th2 cells was dependent on innate cell sources of IL-4/IL-13 and could not be mediated by autocrine IL-4/IL-13. In CD4 T cells, these latter cytokines were critical for efficient IgE production, which may play a role in secondary infections by arming mast cells and contributing to their survival in tissues. These data suggest a model by which type 2-inciting agents, such as worms and allergens, activate dendritic cells to induce Th2 cells in draining lymph nodes, but also

activate a tissue-innate noneosinophil population to produce IL-4/IL-13. These cytokines are presumably critical in recruiting Th2 cells into affected tissues, where these cells subsequently organize two myeloid populations consisting of basophils and eosinophils. Although eosinophils contribute to secondary immune responses through antigen degradation in tissues, defining a primary role for basophils in type 2 immunity will await the development of genetic tools to study these elusive cells.

MATERIALS AND METHODS

Mice. IL-4 reporter mice (4get mice) have been described previously (12). In brief, these mice were generated by introducing an IRES-eGFP construct after the stop codon of IL-4 by homologous recombination, which leads to transcription of a bicistronic IL-4-IRES-eGFP mRNA and translation of both IL-4 and eGFP from the same mRNA. This allows analysis of IL-4-expressing cells in vivo by detection of GFP expression without the need for prior restimulation. IL-4/IL-13-deficient mice were obtained from A.N. McKenzie (Trinity College, Dublin, Ireland; reference 14). IL-5tg mice were obtained from J.J. Lee (Mayo Clinic Arizona, Scottsdale, AZ; reference 20). GATA-1 mutant mice (Δ dbGATA), which lack eosinophils due to a mutation in the GATA-1 binding site of the *gata-1* promoter, were obtained from C. Gerard (Harvard Medical School, Boston, MA; reference 21). TCR- α -deficient mice ($\text{Ca}^{-/-}$ mice; reference 47), STAT6-deficient mice (48), MHC class II-deficient mice (49), and DO11.10 TCR-tg mice (50) were obtained from The Jackson Laboratory. RAG-deficient mice were obtained from Taconic Farms. All mice except 4get/MHC class II $^{-/-}$ mice, which were used on a mixed B6/129 background, were backcrossed at least nine times to BALB/c background and intercrossed with 4get mice to generate the corresponding strains used in this study. Mice were kept under specific pathogen-free conditions in the tg animal facility at the University of California, San Francisco (UCSF), and used for experiments at 6–8 wk of age in accordance with institutional protocols.

Adoptive transfer. Spleens and mesenteric lymph nodes of donor mice were collected in medium (RPMI 1640, 10% FCS) and mechanically dispersed to generate single cell suspensions. Red blood cells were removed by ammonium chloride lysis. For polyclonal T cell transfers into T cell-deficient recipients, CD4 T cells were purified to >95% purity using the MACS CD4 T cell isolation kit and LS columns according to the manufacturer's instructions (Miltenyi Biotec). 10^7 CD4 T cells were transferred in 300 μ l PBS i.v. to recipient mice. For the transfer of OVA-specific TCR-tg cells, total splenocytes from DO11.10/4get/ $\text{Ca}^{-/-}$ mice containing 10^6 KJ1-26 clone-type $^{+}$ CD4 T cells were injected i.v. to BALB/c or IL-4/IL-13-deficient mice. Eosinophils were isolated from the peritoneal cavity of IL-5tg mice, which contains >80% eosinophils, washed twice with PBS, and either transferred directly into recipient mice ($3\text{--}8 \times 10^7$ cells per mouse) or labeled with CFSE before transfer as described previously (51).

In vivo CD4 depletion. Mice were injected i.p. with 1 mg purified anti-CD4 antibody (GK1.5) 2 d before infection with *N. brasiliensis*.

OVA-induced airways inflammation. Mice were given saline or 50 μ g OVA emulsified in 1 mg alum i.p. on days 0, 7, and 14. On days 21, 22, and 23, mice were challenged by intranasal administration of 100 μ g OVA and analyzed on day 24, 1 d after the final challenge. Airway reactivity was measured by the forced oscillation technique. In brief, mice were anesthetized with ketamine/xylazine, and a stub-end adaptor connected to a flexiVent pulmonary mechanics analyzer (Scireq) was inserted into the exposed trachea. Mice were paralyzed (0.1 mg/kg pancuronium, i.p.) and ventilated with 100% oxygen at 150 breaths/min and 9 ml/kg tidal volume. Increasing doses of acetylcholine were then administered through the cannulated tail vein. FACS analysis of dispersed lung cells was performed as described below.

***N. brasiliensis* infection.** Third-stage larvae (L3) of *N. brasiliensis* were recovered from the cultured feces of infected rats, washed extensively in saline (37°C), and injected (500 organisms) into mice subcutaneously at the base of the tail. Mice were provided antibiotic-containing water (2 g/liter neomycin sulfate, 100 mg/l polymyxin B sulfate; Sigma-Aldrich) for the first 5 d after infection. Secondary infections (750 L3 larvae) were performed 4 wk after the primary infection. Worm burden was analyzed 10 d after primary or 7 d after secondary infection after longitudinal dissection of the small intestine.

Flow cytometry. Single cell suspensions of lung and paratracheal lymph nodes were washed in FACS buffer (PBS, 2%FCS, 1 mg/ml sodium azide), incubated with anti-CD16/CD32 Fc block antibody (2.4G2; BD Biosciences) for 5 min at room temperature, and stained with biotinylated anti-IgE (R35-72; BD Biosciences) followed by APC-labeled streptavidin (Invitrogen), PerCP/Cy5.5-labeled anti-CD4 (L3T4; BD Biosciences), and PE-labeled anti-CCR3 (R&D Systems). To detect OVA-specific TCR-tg cells, suspensions were stained with PerCP/Cy5.5-labeled anti-CD4 and APC-labeled KJ1-26 (BD Biosciences). Cells were analyzed on FACSCalibur and LSRII instruments (BD Immunocytometry Systems).

ELISA for serum IgE. Serum IgE was determined by ELISA using the monoclonal antibody B1E3 for coating and the biotinylated monoclonal antibody EM95 for detection.

Bone marrow chimeras. Recipient mice were lethally irradiated using 600 rad administered twice 3 h apart and reconstituted by i.v. transfer of 2×10^6 bone marrow cells from 4get donor mice. Mice were kept on antibiotic-containing water (2 g/liter neomycin sulfate, 100 mg/liter polymyxin B sulfate) and SCID MD tablets (Bioserv) in the specific pathogen-free animal facility at UCSF and used for experiments 7 wk after reconstitution.

Statistical analysis. p-values were calculated using Student's *t* test.

Online supplemental material. Fig. S1 shows lack of recruitment of Th2 cells and eosinophils to the lung and lack of expulsion of *N. brasiliensis* after transfer of eosinophils from IL-5tg mice into IL-4/IL-13/TCR- α -deficient mice that had been reconstituted with purified CD4 T cells from 4get mice before infection with *N. brasiliensis*. It is available at <http://www.jem.org/cgi/content/full/jem.20052448/DC1>.

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